REMARKS

Claims 108, 110-114 and 116-119 are currently pending in the application. Claims 112 and 113, which were allowed per Paper No.: 9 (Office Action issued January 15, 2003), are withdrawn from consideration. It is Applicant's understanding that claims 112 and 113 will be reinstated upon allowance of the claims currently under consideration.

Claims 108, 110, 111, 114 and 116-119 are under consideration and stand rejected under 35 U.S.C. § 103(a) based on a number of positions laid out in detail in the 10/06/04 Final Office Action. Claims 110 and 116 are rejected under 35 U.S.C. § 102(b) over the Nudelman and Windmuller references cited in a previous office action. Applicant respectfully disagrees with the conclusions set forth in that office action.

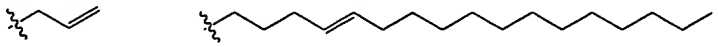
1. Rejections under 35 U.S.C. § 102(b)

A. 35 U.S.C. § 102(b) rejection over Windmüller *et al.* (Tetrahedron Letters, 1994, Vol. 35, pp. 7927-7930).

The Examiner maintains that Windmüller *et al.* anticipates claims 110 and 116 because it discloses the chemical structure of claims 110 and 116 where R is ceramide or an Sp linking group $-(CH_2)_8CO_2Me$ (citing structure 2c on page 7929).

To dispel any misconception, Applicant's arguments regarding the carrier applied to the recited –Linker-$(Crosslinker)_q$-Carrier element, not to the definition of R taken in its entirety. It was clear to Applicant that ceramide did not fall within the scope of "H, substituted or unsubstituted allyl or an amino acyl moiety." Accordingly, Applicant focused the argument on the –Linker-$(Crosslinker)_q$-Carrier element, which Applicant believed was the point of contention. Clearly, the Examiner agrees with Applicant that ceramide does not read on –Linker-$(Crosslinker)_q$-Carrier, therefore Applicant's line of argument regarding the carrier is now moot.

However, the Examiner appears to believe that ceramide falls within the scope of substituted or unsubstituted allyl. Applicant strongly disagrees. Ceramide, as depicted on page 7927 of the Windmuller reference, is a substituted octadec-5-enyl moiety, not a substituted allyl moiety.



Allyl Octadec-5-enyl

R = optionally substituted allyl means that ***one*** carbon atom separates the carbohydrate from the allyl double bond.

R = ceramide (i.e., optionally substituted octadec-5-enyl) means that ***three*** carbon atoms separate the carbohydrate from the ceramide double bond.

Therefore, the Windmüller reference cannot anticipate claim 110.

Claim 116 is directed to compositions comprising an inventive compound *and an immunological adjuvant and/or a pharmaceutically acceptable carrier.* The Windmüller reference does not teach compositions comprising an immunological adjuvant and/or a pharmaceutically acceptable carrier. Therefore, the Windmüller reference cannot anticipate claim 116.

B. 35 U.S.C. § 102(b) rejection over Nudelman *et al.* (The Journal of Biological Chemistry, 1986, Vol. 261, pp. 11247-11253).

The Examiner states that Nudelman et al. teach the chemical structure of claims 110 and 116 wherein R is ceramide and the indices are r=1, m=0 and n=1 (citing structure 2c on page 7929).

Applicant respectfully submits that Nudelman cannot anticipate claim 110 for reasons discussed in section A above (*i.e.*, R, as recited in instant claim 110, does not encompass Ceramide). Likewise, the Nudelman reference cannot anticipate claim 116 because Nudelman does not teach compositions comprising an immunological adjuvant and/or a pharmaceutically acceptable carrier. In addditon, R, as recited in instant claim 116, does not encompass Ceramide.

In view of the remarks above, Applicant respectfully requests that the 102(b) rejections of record be withdrawn.

2. Rejection under 35 U.S.C. § 103(a)

On page 3, first paragraph, of the 10/06/04 Final Office Action, the Examiner has maintained the rejection of claims 108, 110, 111, 114 and 116-119 under 35 U.S.C. § 103(a) as being allegedly unpatentable over Etlinger (EP 429816) (hereinafter "Etlinger"), in view of Sytokowski (WO 95/25746) (hereinafter "Sytokowski"), in further view of Nudelman *et al.* (The Journal of Biological Chemistry, 1986, Vol. 261, pp. 11247-11253) (hereinafter "Nudelman") and in further view of Kaizu *et al.* (The Journal of Biological Chemistry, 1986, Vol. 261, pp. 11254-11258) (hereinafter "Kaizu"). The Examiner further concludes in the paragraph bridging pages 5 and 6 of that Office Action, with a *prima facie* obviousness rejection based on the Etlinger, Sytokowski, Nudelman and Kaizu references.

However, the Examiner also discusses the Windmuller reference (page 4 last paragraph of the 10/06/04 Office Action) and concludes with a *prima facie* obviousness rejection based on the Etlinger, Sytokowski, Nudelman, Kaizu and Windmuller references (paragraph bridging pages 4 and 5).

Since the Examiner has not clarified whether she meant to include the Windmuller reference in the combination of cited references to support her 103(a) rejection, Applicant will address the stated rejection as if it were two separate 103(a) rejections, and demonstrate that, whether or not the Windmuller reference is relied on, the stated 103 rejection is improper.

The legal standard for establishing a *prima facie* case of obviousness requires that three basic criteria be met: (1) there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one skilled in the art, to modify the reference or to combine reference teachings; (2) there must be a reasonable expectation of success in the modification or in the combination; and (3) the prior art reference must teach all

the claim limitations. All three requirements must be met to establish a *prima facie* case of obviousness. In addition, the teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art and not based on applicant's disclosure (MPEP 706.02(j)). Applicant respectfully submits that at least one of the three requirements has not been met for the reasons that follow.

A. Rejection under 35 U.S.C. § 103(a) over Etlinger, in view of Sytokowski, in further view of Nudelman and in further view of Kaizu.

The Examiner alleges that (1) Etlinger teaches a method for inducing a humoral response comprising the administration of an antigen which comprises a B-cell epitope linked to a carrier protein, where the carrier protein comprises a T-helper cell epitope (citing page 2 lines 22-25, page 3 lines 39-49 and page 4 lines 23-34); (2) Sytokowski teaches that for glycoproteins, a heterobifunctional cross linking reagent can be attached to a carbohydrate moiety and linked to a primary amine within a peptide (citing page 7 lines 17-28), specifically that 4-(N-maleimidomethyl)cyclohexane-1-carboxyl-hydrazide can be used as a linking agent; (3) Nudelman teaches the trifucosylnonasylceramide structure 2, as a major component of Le^y-active components detected in human colonic carcinoma cases; (4) Kaizu teaches that administration of the trifucosylnonasylceramide to mice with Salmonella minnesota as adjuvant produced the IgM KH1 antibody with a novel specificity for trifucosylnonasylceramide. The Examiner then concludes that it would have been prima facie obvious at the time the present invention was made to use the trifucosylnonasylceramide structure identified by Kaizu as the B-cell epitope in the method taught by Etlinger and attach said trifucosylnonasylceramide to the carrier protein or peptide by the linker taught by Sytokowski.

Applicant respectfully traverses the Examiner's rejection and asserts that a *prima facie* case of obviousness has not been established because there is no suggestion or motivation in any of the cited references to link the structure as provided by Kaizu *et al.* (e.g., a *ceramide* structure)

to a linker or carrier molecule, and furthermore, there is no reasonable expectation of success to attach the minute amounts of the structure provided by Kaizu *et al.* to a carrier molecule. In addition, the combination of references would not allow one skille din the art to achieve the claimed invention.

As the Examiner has recognized, Kaizu *et al.*, in attempting to develop a diagnostic tool that will identify KH-1 in the presence of other related antigens, provide the antigen in the form of a *ceramide* (e.g., a glycolipid isolate). As shown in the companion paper, Nudelman et al., also cited by the Examiner, this ceramide is provided in *extremely minute* quantities (e.g., 100-150 micrograms, as shown in the Supplemental Materials (page 11252) in the cited Nudelman reference).

Significantly, neither Nuldelman nor Kaizu teach any synthetic methodology for the attachment of ceramide structures to carrier molecules directly or through a linker, nor do they teach methodology for the modification of ceramide structures to enable attachment to carrier molecules. Contrary to the Examiner's assertion, Applicant additionally asserts that it would not be possible to effectively attach the trifucosylnonasylceramide as provided by Kaizu *et al.* to carrier proteins. Specifically, in order to effect attachment and generate the presently claimed compounds, it would first be necessary to cleave the ceramide, or subject it to reaction conditions, to provide a suitable functionality for attachment (e.g., an aldehyde). However, conditions that would be able to effect cleavage of the ceramide would also compromise other glycosidic bonds, thus destroying the carbohydrate antigen, and rendering it unsuitable for its intended purpose, or any other use requiring a functional carbohydrate determinant (e.g., Kaizu *et al.*). In addition, these modifications are not suggested, much less taught, in any of the cited references. Additionally, given the quantities of materials as provided by Kaizu *et al.* and Nudelman et al. (microgram quantities), it would be virtually impossible to modify the ceramide structure for attachment to carrier proteins. Applicant respectfully submits that reasonable quantities of materials having suitable conjugation functionalities can only realistically be provided by synthetic methods.

In addition to the fact that there are no known methods in the art for the direct attachment of the ceramide structure to a carrier protein without some sort of modification, and any attempts to cleave the entire ceramide portion would result in destruction of the antigen (thus rendering it useless for its intended purpose), Applicant further asserts that even if there were known synthetic methods to effect cleavage of the entire ceramide structure without compromising the carbohydrate, or if there were general methods available for the *direct* attachment of the ceramide structure, the amount of material provided by Kaizu *et al.* and Nudelman et al. is so minute that these synthetic modifications would be virtually impossible. Additionally, even if the conjugation was somehow successful despite the minute quantities of material, the amount of material finally generated (presumably less than microgram quantities) would not be enough material to elicit any kind of useful immune response.

Sytokowski teaches carbohydrate-specific heterobifunctional reagents for cross-linking glycoproteins (via attachment to a carbohydrate of the glycoprotein) with other macromolecular constructs (e.g., polypeptides and proteins). The Sytokowski references discloses 4-(N-maleimidomethyl) cyclohexane-1-carbonyl-hydrazide-HCl as an example of such carbohydrate-specific heterobifunctional reagent (page 15 lines 23-25). Applicant respectfully points out, however, that such reagents (hydrazine reagents) react with the *oxidized form* of carbohydrate moieties. As described in "Advanced Organic Chemistry", March, J. John Wiley & Sons, 2001, 5th ed., section 16-19 pp. 1192-1193 (Exhibit A), hydrazines react with aldehydes or ketones. As shown in Figure 13 of Applicant's disclosure, 4-(N-maleimidomethyl) cyclohexane-1-carbonyl-hydrazine does not react with a carbohydrate lacking aldehyde or ketone groups. Thus, attachment of a hydrazine carbohydrate-specific heterobifunctional cross-linker, such as those taught by Sytokowski, would require an aldehyde/ketone-containing carbohydrate, or prior oxidation of the carbohydrate moiety (e.g., $NaIO_4$) to introduce a functional group suitable for covalent attachment to the hydrazine moiety (e.g., an aldehyde). Applicant points to U.S. Patent 6,359,118 (Exhibit B) disclosing a method for carbohydrate cross-linking of glycoproteins using a diamine reagent such as 4-(N-maleimidomethyl) cyclohexane-1-carbonyl-hydrazide-HCl (See

column 8 lines 52-53 and lines 60-65). Additional support can be found in Uptima Product Description on SH and CHO reactive crosslinkers (Exhibit C – See, for example page 2, fourth bullet point "*The hydrazide group reacts specifically with aldehyde, forming a stable hydrazone bond. [...] Aldehydes are present in reducing oses, or can be generated from cis-dol found notably in carbohydrates by specific oxidases such as galactose oxidase, or by mild oxidation with 10mM $NaIO_4$ at RT in the dark*"). Therefore, Applicant submits that one of ordinary skill in the art would not be motivated to attach Kaizu's trifucosylnonasylceramide to a carrier protein or peptide using the linker taught by Sytokowski, as the Examiner suggests, because it implies initial oxidation of the carbohydrate moiety, which would destroy the carbohydrate antigen, and render it unsuitable for its intended purpose, or any other use requiring a functional carbohydrate determinant (e.g., Kaizu *et al.*). Even if Sytokowski taught or suggested *indirect* attachment of the crosslinkers to a carbohydrate via a linker present on the carbohydrate (e.g., ceramide), the combination of cited references would still not support a prima facie case of obviousness because there would be no expectation of success in the combination. Specifically, chemical modification of the ceramide moiety to introduce a functional group suitable for reaction with Sytokowski's heterobifunctional hydrazide linkers would also compromise other glycosidic bonds, thus destroying the carbohydrate antigen. Additionally, given the quantities of materials as provided by Kaizu *et al.* and Nudelman et al. (microgram quantities), it would be virtually impossible to modify the trifucosylnonasylceramide of Kaizu for attachment to carrier proteins using the linker taught by Sotokowski.

Clearly, as discussed above, there would be no reasonable expectation of success that one of ordinary skill in the art would be able to attach a carrier protein to a trifucosylnonasylceramide structure as taught by Kaizu *et al.* using methodology that would require a suitable reactive functionality such as an aldehyde or ketone (which, clearly, the ceramide structure lacks). Furthermore, there would be no reasonable expectation of success that one would be able to remove the entire ceramide structure to provide a suitable functionality because any attempt to cleave or remove the entire ceramide structure would require using conditions that would

compromise the other glycosidic bonds, thus destroying the carbohydrate antigen and rendering it unsuitable for its intended purpose. Finally, because Kaizu *et al.* (or any researchers providing such ceramide structures isolated from natural sources) are only able to obtain the ceramide structure in such *minute* quantities, there would be no reasonable expectation of success to perform any synthetic transformations (e.g., cleavage or modification), let alone isolate, characterize, and utilize any resultant constructs. The combination of Kaizu *et al.*, Nudelman et al. and Sotokowski, in view of Etlinger et al., as suggested by the Examiner, would only result in failed reaction products (likely uncharacterizable because of the minute quantities) because the methodology applied in Sotokowski which utilizes a reactive hydrazine functionality is not compatible with the unreactive ceramide structure or the trifucosylnonasyl construct. Additionally, the minute quantities provided by Kaizu *et al.* would preclude being able to perform any synthetic modifications to generate reasonable quantities of material for the generation of the novel compounds as taught and used in the presently claimed invention.

In summary, none of the Etlinger, Sytokowski, Nuldelman and Kaizu references provide any teaching or suggestion to modify any of the cited references to achieve the claimed invention, much less teaching or suggestion as to how this might be accomplished. The Sytokowski reference alone would discourage any person skilled in the art to combine Sytokowski's teachings with that of Etlinger, Nuldelman and Kaizu, because the combination would not achieve the claimed invention (the use of the carbohydrate specific heterobifunctional hydrazines as taught by Sotokowski to link Kaizu's trifucosylnonasyl-ceramide to a carrier protein or peptide would result in destruction of the carbohydrate antigen, and render it unsuitable for its intended purpose).

In view of the arguments presented above, Applicant respectfully submits that claims 108, 110, 111, 114 and 116-119, as pending, are nonobvious over Etlinger, Sytokowski, Nudelman *et al.* and Kaizu *et al.* Applicant thus respectfully requests that the Examiner withdraw the stated rejection under 35 U.S.C. § 103.

B. Rejection under 35 U.S.C. § 103(a) over Etlinger, in view of Sytokowski, in further view of Nudelman, in further view of Kaizu and in further view of Windmuller.

The Examiner relies on the Kaizu and Nudleman references as motivation to make antibodies to the claimed trifucosylnonasyl epitope. The Examiner states that the Sytokowski reference was relied upon for the teaching of heterobifunctional crosslinkers which are used to attach carbohydrate groups to proteins. The Examiner states that the Etlinger reference teaches a method for inducing a humoral response comprising the administration of an antigen which comprises a B-cell epitope linked to a carrier protein, where the carrier protein comprises a T-helper cell epitope, citing page 2 lines 22-25, page 3 lines 39-49 and page 4 lines 23-34. The Winmuller reference was relied upon for the teaching of the synthesis of compound **5** bearing a $-(CH_2)_8CO_2Me$ moiety, which the Examiner asserts would allow for the attachment to a carrier protein using the reagent 4-(N-maleimidomethyl)cyclohexane-1-carboxyl-hydrazide, as taught by Sotokowski. The Examiner then concludes that the skilled practitioner would have been motivated to use the trifucosylnonasylceramide structure identified by Kaizu as the B-cell epitope in the method taught by Etlinger and attach said trifucosylnonasylceramide to the carrier protein or peptide by the linker taught by Sytokowski, through the teachings of Kaizu on the ability of the trifucosylnonasylceramide to elicit IgM antibodies, the teachings of Sytokowski on the use of heterobifunctional linkers to link carbohydrates of glycoproteins to primary amines of proteins, and the teachings of Windmuller on the chemical synthesis of building block **5** bearing a $-(CH_2)_8CO_2Me$ moiety.

Applicant respectfully traverses the Examiner's rejection and asserts that a *prima facie* case of obviousness has not been established because there is no suggestion or motivation in any of the cited references to link the structure as provided by Kaizu (e.g., a *ceramide* structure) or by Windmuller (e.g., compound **2c** bearing a $-(CH_2)_8CO_2Me$ moiety) to a linker or carrier molecule, and furthermore, there is no reasonable expectation of success to attach the structure provided by Kaizu *et al.* or the $MeOC(=O)-(CH_2)_8$-containing trifucosylnonasyl construct taught by Windmuller *et al.* to a carrier molecule via a linker taught by Sytokowski.

Arguments in support of Applicant's position that the cited references do not make prima facie obvious the attachment of Kaizu's trifucosylnonasylceramide compound to a carrier protein or peptide are provided in Section A above. Specifically, in order to effect attachment and generate the presently claimed compounds, it would first be necessary to cleave the ceramide, or to subject it to reaction conditions, to provide a suitable functionality for attachment. However, conditions that would be able to effect cleavage of the ceramide would also compromise other glycosidic bonds, thus destroying the carbohydrate antigen, and rendering it unsuitable for its intended purpose, or any other use requiring a functional carbohydrate determinant (e.g., Kaizu *et al.*).

With respect to the Examiner's statement that the $-(CH_2)_8CO_2Me$ moiety would allow for the attachment (presumably of Windmuller's compound **2c**?, although the Examiner does not specifically state this) to a carrier protein using the reagent 4-(N-maleimidomethyl)cyclohexane-1-carboxyl-hydrazide, as taught by Sotokowski, Applicant maintains that there is no teaching or suggestion in any of the cited references of attaching Windmuller's $-(CH_2)_8CO_2Me$ spacer to a carrier protein via a 4-(N-maleimidomethyl)-cyclohexane-1-carboxyl-hydrazide crosslinker, much less teaching of how this might be accomplished. In fact, Sytokowski *teaches away* from this very proposition. As discussed in Section A above, the carbohydrate-specific heterobifunctional hydrazine reagents taught by Sytokowski attach *directly* to the carbohydrate moieties (more specifically with the *oxidized form* of carbohydrate moieties). Again, one of ordinary skill in the art would not be motivated to attach Windmuller's $-(CH_2)_8CO_2Me$ spacer to a carrier protein or peptide using the linker taught by Sytokowski, as the Examiner suggests, because Sytokowski's teachings imply attachment on the carbohydrate *directly* (via initial oxidation of the carbohydrate moiety, which would destroy the carbohydrate antigen, and render it unsuitable for its intended purpose, or any other use requiring a functional carbohydrate determinant (e.g., Kaizu *et al.*)).

Nowhere in the cited references is it suggested, much less taught, that a carbohydrate–$(CH_2)_8CO_2Me$ construct (such as Windmuller's compound **2c**) can be linked to a protein or

peptide carrier by the hydrazine linkers of Sytokowski whereby covalent attachment takes place via the $-(CH_2)_8CO_2Me$ moiety. In fact, Applicant submits that such attachment would not be possible. As seen in Exhibit A, heterobifunctional hydrazine reagents such as those taught by Sytokowski (e.g., 4-(N-maleimidomethyl) cyclohexane-1-carbonyl-hydrazide-HCl) react with with aldehydes or ketones, *not carboxylic esters*. Thus, attachment of the $-(CH_2)_8CO_2Me$ moiety to a hydrazine heterobifunctional cross-linker would require chemical transformation (i.e., reduction) of the carboxylic ester moiety to an aldehyde. Such a transformation is typically carried out with a hydride reagent (e.g., DIBAL-H, $NaAlH_4$, $LiAlH_4$-Et_2NH), which would likely affect the carbohydrate hydroxyl and –NHAc groups. Specifically, the hydroxyl groups would react/interfere with the reducing agent, and/or the –NHAc group would get reduced, thus modifying the carbohydrate antigen, with no reasonable expectation that the modified carbohydrate would be suitable for its intended purpose. The mere fact that the above discussed considerations are not addressed in any of the cited references is evidence that the Examiner's alleged reconstruction of the claimed invention by combining the teachings of Etlinger, Sytokowski, Nuldelman, Windmuller and Kaizu was simply not considered in any of the cited references. In fact, the combination of cited references would not allow one skilled in the art to achieve the claimed invention. Applicant submits that the skilled practitioner would conclude the undesirability of the cited combination from the Sytokowski reference alone (the use of the carbohydrate specific heterobifunctional hydrazines as taught by Sotokowski to link Kaizu's trifucosylnonasyl-ceramide or Windmuller's trifucosylnonasyl–$(CH_2)_8CO_2Me$ construct to a carrier protein or peptide would result in destruction of the carbohydrate antigen, and render it unsuitable for its intended purpose).

In summary, none of the Etlinger, Sytokowski, Nuldelman, Windmuller and Kaizu references provide any teaching or suggestion to modify any of the cited references to achieve the claimed invention, much less teaching or suggestion as to how this might be accomplished. There is also no motivation in the combination. Accordingly, Applicant maintains that the Examiner has failed to establish a *Prima Facie* case of obviousness. Therefore, claims 108, 110,

111, 114 and 116-119 cannot be held obvious over any one or more of Etlinger, Sytokowski, Nuldelman, Windmuller and Kaizu references. Applicant respectfully requests that the stated 103 rejection be withdrawn.

CONCLUSION

Applicant thanks the Examiner for his/her time and consideration. If a telephone conversation would help clarify any issues, or help expedite prosecution of this case, Applicant invites the Examiner to contact the undersigned at (617) 248-5150.

It is not believed that fees are required, beyond those which may otherwise be provided for in documents accompanying this paper. However, in the event that any additional fees required for consideration of this paper, such fees are authorized to be charged to our Deposit Account No. 03-1721.

Respectfully submitted,

Date: January 6, 2005

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Dated: January 6, 2005

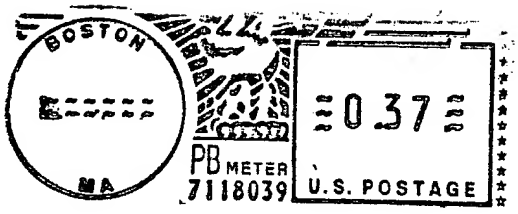
The Patent and Trademark Office stamping sets forth the filing and receipt date of the documents listed below for patent application identified as follows:

Applicant:	Danishefsky *et al.*	Examiner:	Karen A. Canella
Serial No.:	09/833,327	Art Unit:	1642
Filed:	April 12, 2001		
For:	COLON CANCER KH-1 AND N3 ANTIGENS		

1. Transmittal Letter (2 pages);
2. Response under 37 C.F.R. § 1.116 (13 pages);
3. Exhibit A - "Advanced Organic Chemistry", March, J. John Wiley & Sons, 2001, 5th ed., section 16-19 pp. 1192-1193 (3 pages, including cover page);
4. Exhibit B - U.S. Patent 6,359,118 (22 pages, including cover page);
5. Exhibit C - Uptima Product Description on SH and CHO reactive crosslinkers (3 pages, including cover page); and
6. Return Postcard.

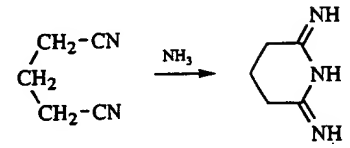
Attorney: BHJ/NML Case No.2003080-0081 (SK-719-Z)

3786327v1



CHOATE, HALL & STEWART
EXCHANGE PLACE
53 STATE STREET
BOSTON, MA 02109
ATTN: PATENT DEPARTMENT

suitable chain length can give imidines:[200]



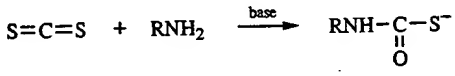
Primary and secondary amines can be used instead of ammonia, to give substituted amidines, but only if the nitrile contains electron-withdrawing groups; for example, Cl_3CCN gives the reaction. Ordinary nitriles do not react, and, in fact, acetonitrile is often used as a solvent in this reaction.[201] However, ordinary nitriles can be converted to amidines by treatment with an alkylchloroaluminum amide, $MeAl(Cl)NR_2$ (R = H or Me).[202] The addition of ammonia to cyanamide NH_2CN gives guanidine $(NH_2)_2C{=}NH$.

If water is present, and a ruthenium complex catalyst is used, the addition of a primary or secondary amine to a nitrile gives an amide: $RCN + R'NHR'' + H_2O \rightarrow RCONR'R'' + NH_3$ (R″ may be H).[203]

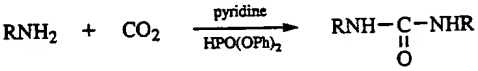
OS I, 302 (but also see OS V, 589); IV, 245, 247, 515, 566, 769. See also OS V, 39.

16-18 *The Addition of Amines to Carbon Disulfide and Carbon Dioxide*

S-METALLO-*C*-ALKYLAMINO-ADDITION



Salts of dithiocarbamic acid can be prepared by the addition of primary or secondary amines to carbon disulfide.[204] This reaction is similar to **16-9**. Hydrogen sulfide can be eliminated from the product, directly or indirectly, to give isothiocyanates (RNCS). Isothiocyanates can be obtained directly by the reaction of primary amines and CS_2 in pyridine in the presence of DCC.[205] In the presence of diphenyl phosphite and pyridine, primary amines add to CO_2 and to CS_2 to give, respectively, symmetrically substituted ureas and thioureas:[206]

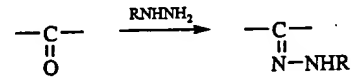


OS I, 447; III, 360, 394, 599, 763; V, 223.

E. *Other Nitrogen Nucleophiles*

16-19 *The Addition of Hydrazine Derivatives to Carbonyl Compounds*

HYDRAZONO-DE-OXO-BISUBSTITUTION

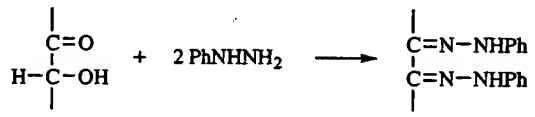


The product of condensation of a hydrazine and an aldehyde or ketone is called a *hydrazone*. Hydrazine itself gives hydrazones only with aryl ketones. With other aldehydes and ketones, either no useful product can be isolated, or the remaining NH_2 group condenses with a second mole of carbonyl compound to give an *azine*. This type of product is especially important for aromatic aldehydes:

$$ArCH{=}N{-}NH_2 + ArCHO \longrightarrow ArCH{=}N{-}N{=}CHAr$$

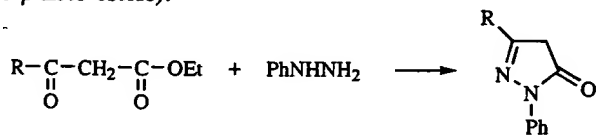
An azine

However, in some cases azines can be converted to hydrazones by treatment with excess hydrazine and NaOH.[207] Arylhydrazines, especially phenyl, *p*-nitrophenyl, and 2,4-dinitrophenyl,[208] are used much more often and give the corresponding hydrazones with most aldehydes and ketones.[209] Since these are usually solids, they make excellent derivatives and are commonly employed for this purpose. Cyclic hydrazones are also known,[210] as are conjugated hydrazones.[211] α-Hydroxy aldehydes and ketones and α-dicarbonyl compounds give *osazones*, in which two adjacent carbons have carbon–nitrogen double bonds:

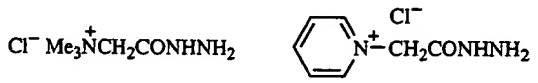


An osazone

Osazones are particularly important in carbohydrate chemistry. In contrast to this behavior, β-diketones and β-keto esters give *pyrazoles* and *pyrazolones*, respectively (illustrated for β-keto esters):



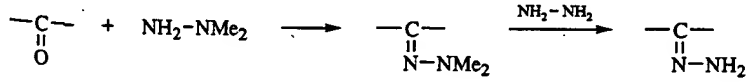
Other hydrazine derivatives frequently used to prepare the corresponding hydrazone are semicarbazide ($NH_2NHCONH_2$), in which case the hydrazone is called a semicarbazone, and *Girard's reagents T and P*, in which case the hydrazone



Girard's reagent T Girard's reagent P

is water soluble because of the ionic group. Girard's reagents are often used for purification of carbonyl compounds.[212]

Simple N-unsubstituted hydrazones can be obtained by an exchange reaction. The *N,N*-dimethylhydrazone is prepared first and then treated with hydrazine:[213]



No azines are formed under these conditions.

Uptima

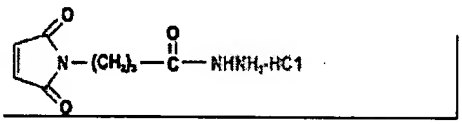
FT-UPG9910

MPH, MCH(EMCH), KMUH, MPBH

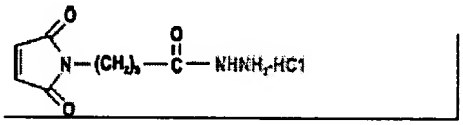
SH and CHO reactive Crosslinkers

Product Description

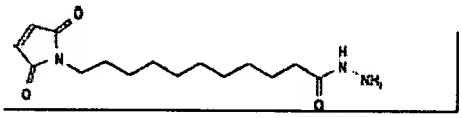
Catalog number: UPG9909A, 50mg
Name: **MPH**
Formula : MaleimidoPropionic acid Hydrazide, HCl
$C_8H_{12}N_3O_3Cl$, M.W.= 233.65



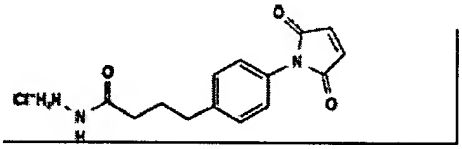
Catalog number: UPG9910A, 50mg
Name: **MCH, EMCH**
Formula : MaleimidoCaproic acid Hydrazide, HCl
$C_{10}H_{16}N_3O_3Cl$, M.W.= 261.71



Catalog number: UPL7722A, 50mg UPL7722B, 100mg
Name: **KMUH**
Formula : N-(k-Maleimidoundecanoic acid)hydrazide
$C_{15}H_{25}N_3O_3$ M.W.= 295.4



Catalog number: UP09835A, 100mg UP09835B, 50mg
Name: **MPBH**
Formula : 4-(4-N-MaleimidoPhenyl)butyric acid Hydrazide.HCl
M.W.= 309.5



Storage : +4°C (long term: -20°C) , protect from moisture and light. (L)

General Information

Cross-linkers are chemical reagents used to conjugate molecules together by a covalent bound. Several atoms separate the 2 molecules, forming the ' . The conjugate associates the characteristics and biological activities of each components.

Cross-linkers have become important tools for the preparation of conjugates used in a lot of immunotechnologies, and for protein studies (structure, interactions, activity, degradation...). To that point, **heterobifunctionnal crosslinkers** are probably the most interesting, because they present 2 reactivities that allow the conjugation of molecules in a defined manner, avoiding notably the formation of dimeres and polymeres. The choice of reactivitie is determinant for the design of the right conjugate. An important other thing to consider is the nature and length of the spacer.

Uptima offers 3 sulfhydryl and aldehyde reactive cross-linkers of high quality to answer the needs of coupling biomolecules for biological and immunoassays like (other cross-linkers are available): MPH, MCH and KMUH differ by the length of the spacer, and are used classically to cross-link a thiolated protein to a carbohydrate, allowing well directed conjugation.

Applications involve notably glycated or reduced proteins, peptides, nucleic acids:

- Obtention of immunogens carrier-hapten
- Obtention of labeled affine probes: for example, antibodies coupled to enzyme for immunoblotting, fluorophore-peptides conjugates for the study of receptors, enzyme-drugs for using as tracers in ELISA...
- Obtention of oligomeric conjugates : conjugates of glycolipids for immunization, structural studies...
- Immobilization of ligands: grafting haptens onto cells, gels or functionalized supports...
- Obtention of biologically active conjugates: specific antibody coupled to drugs for immunotargetting techniques, immunotoxins, ...

-

This sheet describes cross-linkers that contains a reactivity toward **sulfhydryls**, through the maleimide group, and a reactivity toward **carbohydrates**, through the hydrazide group.

Scientific and Technical Information

- The **spacer arm** span 4 atoms (MPH),
  6 atoms =11.8 Å length (MCH),
  11 atoms = 19.0 Å (KMUH).
  It is linear but flexible, allowing the interaction of conjugated molecules on both sides.
  MPBH spacer is 17.9 Å long, and contains a aromatic cycle that reduces slightly the flexibility. It is also more immunogenic.

- MPBH **solubility** is 620 mM in DMSO, 1500 mM in DMF and around 1 M in aqueous buffers.

- The **maleimide** group reacts very specifically with sulfhydryls –SH at neutral pH >6.5. The reaction is rapid (a few minutes for cystein), but in the absence of –SH, it is well stable. The hydrolysis forming maleimic acid becomes noticeable when pH go up 8.0, where the reactivity with amines begins to be possible. In usual conditions, pH6.5-7.5, one should start with a ratio of 10-20 moles of maleimide per mole of protein. With SH-peptides, a molar 1:1 incubation ratio allows usually almost 1:1 coupling.

Sulfhydryls are available in some proteins and peptides (often synthetized with a N-terminus Cysteine), or can by generated by reduction with DTT (#UP28425) or introduced with Iminothiolane (#UP42425) or SATA (#UP84235)

- The **hydrazide** group reacts specifically with aldehyde, forming a stable hydrazone bond.
  R-CHO + N2-NH-R' → R-CH=N-NH-R'

Aldhehydes are present in reducing oses, or can be generated from cis-diol found notably in carbohydrates by specific oxidases such as galactose oxidase, or by mild oxidation with 10mM NaIO4 at RT in the dark (Chamow 1978):

R-CH(OH)-CH(OH)-R + NaIO4 → R-CHO

Rem: Hydrazide also reacts with carboxylic acids in the presence of EDAC (#UP520050)
R-COOH + Hydrazide-R' + EDAC → R-CO-NH-NH-CO-(CH2)4-R'

Rem: As hydrazone bond is not enough stable at very low pH, this can be converted to hydrazine by reaction with NaHBH4 for some applications (O'Shannessey 1990).

- The **reaction scheme** of the conjugation should be designed depending on each application. The ratios of cross-linker to molecules and reaction steps should be determined in each step for each application.

One could for example activated first the SH bearing molecule, because maleimide react quickly and very specifically at pH 6.5-7.5, then add the aldehyde bearing molecule. Desalting of by product may be performed by dialysis (CelluSep) or gelfiltration.

When a molecule bears both chemical groups, one should choose , in order to avoid the formation of dimeres, either to block undesired SH on molecule for the activation step, or of the excess of maleimide for the coupling step, or to invert the steps. At the opposite, EDTA can be added in buffers to prevent the reoxidation of SH into dissulfides (Ishikawa 1983).

Specific protocol can be found in the literature (Chamow 1992): IgG can be oxidized for creating CHO groups that can be activated by the hydrazide group of the cross-linker (ratio 16:1, 2H at room temperature in acetate 0.1M pH5.5). After desalting, the maleimide that is relatively stable during the activation step, reacts with a SH-bearing protein like b-galactosidase or hemoglobin (ratio 1:1, 1H at room temperature in PBS pH7.0). The cross-linking of the antibody through their carbohydrate residue, located on the Fc portion, allows to maintain excellent immunological recognition (antigen binding).

Other Information

For use *in vitro* only, not for diagnostic.

Literature

Trail, P.A., *et al.* (1993). Cure of xenografted human carcinomas by BR96-doxorubicin immunoconjugates. *Science* **261**, 212-215.
Chamow, S.M., Kogan, T.P., Peers, D.H., Hastings, R.C., Byrn, R.A. and Askenaszi, A. (1992). Conjugation of soluble CD4 without loss of biological activity via a novel carbohydrate-directed cross-linking reagent. J. Biol. Chem. 267(22), 15916-15922.
Chamow S.M. et al, Biochem J.,1978, 173, 723-

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